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14. ABSTRACT Tumor-associated macrophages may comprise up to 50% of the tumor mass in breast cancer and are capable of producing estrogen and angiogenic cytokines that regulate the growth and angiogenesis of breast cancer. The purpose of this study is to determine whether intratumoral injection of liposome-encapsulated dichloromethylene diphosphonate (clodronate), a potent macrophage-depleting agent, can deplete tumor-associated macrophages in a murine breast cancer model, and whether depletion of tumor-associated macrophages has any effect on the tumor growth. The breast cancer model was established in BALB/c mice by subcutaneous injection of estrogen receptor-positive murine mammary tumor cells (4T1). Two weeks after injection of 4T1 cells, tumor-bearing mice were divided into 3 groups. The first group served as control with no further injection. The second group was injected intratumorally with liposomes containing clodronate. The third group was injected with liposomes containing phosphate-buffered saline. The tumor size was measured every 2-3 days using a caliper. At 1, 3, 5, 7 and 9 days after liposome injection, tumors were harvested, fixed, and immuno-stained with antibodies to macrophage-specific markers (F4/80 and Mac-1) to quantify the number of macrophages. The infiltrating macrophages were quantified using Chalkley Counting method with a 25-point array reticle. Results showed that the intratumoral injection of 10, 30 or 60 μ l liposome-encapsulated clodronate had no effect on the tumor growth and tumor-associated macrophages in this murine 4T1 breast cancer model. Whether liposome-encapsulated clodronate at a higher dosage has any effect needs further investigation.				
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INTRODUCTION

Tumor-associated macrophages (TAM) are one major component of tumor stroma. In breast cancer, TAM may comprise up to 50% of the tumor mass (1) and are capable of producing estrogen and angiogenic cytokines such as vascular endothelial growth factor and tumor necrosis factor alpha, that regulate the growth and angiogenesis of breast cancer (1-3). There is a positive correlation between high vascular grade and increased macrophage index, and a strong relationship between increased macrophage counts and reduced relapse-free survival as well as reduced overall survival in patients with breast cancers (3). In addition, the accumulation of TAM with thymidine phosphorylase is a potent prognostic indicator of early relapse in primary breast cancer (4). Despite the potential importance of TAM, current treatments of breast cancer have been primarily directed at breast cancer cells. Depletion of TAM may significantly improve the outcome of breast cancer therapy. The purpose of this study is to investigate whether depletion of TAM can slow breast tumor growth and improve the outcome of breast cancer treatment. Depletion of breast cancer TAM will be achieved using intra-tumoral injection of liposome-encapsulated dichloromethylene diphosphonate (clodronate), a specific and potent macrophage-depleting agent (5-8). Using a well established murine 4T1 breast cancer model (9,10), our specific objectives are: 1) To determine whether intra-tumoral injection of liposome-encapsulated clodronate can deplete TAM, 2) To determine the effect of depleting TAM on the tumor growth and angiogenesis, and 3) To determine the effect of depleting TAM on the outcome of chemotherapy.

BODY

Two tasks are defined in the study: Task 1, Establish optimum conditions for depleting TAM using liposome encapsulated clodronate; and Task 2, Determine effects of macrophage depletion on breast cancer growth and angiogenesis, and the outcome of chemotherapy. There are 4 stages proposed to accomplish Task 1: a, Establish the culture of 4T1 breast cancer cells and breast cancer model in BALBc mice; b, Prepare liposome-encapsulated clodronate; c, Treat (intra-tumoral injection) tumor-bearing mice with liposome-encapsulated clodronate; and d, Process tissue for immunohistochemical staining of macrophage-specific marker and quantify macrophages using Chalkley point counting method (11).

1. Materials and Methods

Female BALB/c mice between 18-20 grams were purchased from Charles River Laboratories, and quarantined for 1-2 week before the experiments. Estrogen receptor-positive murine mammary tumor cells (4T1) were obtained from the American Type Culture Collection, and cultured in RPMI 1640 medium containing 10% FBS and 100 Units/ml penicillin and 100 μ g/ml of streptomycin. Cells were collected by centrifugation and washed with the culture medium. Cells were quantified and adjusted to 10^6 cells/ml before being injected into the animals. Liposomes containing clodronate or PBS as control (clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany). The liposomes were warmed to 37°C before injection. Affinity-purified monoclonal antibodies to mouse macrophage marker F4/80 and Mac-1 were purchase from eBioscience. Immunohistochemistry staining kit (Vectastain ABC Kit) was purchased from Vector Laboratories.

Mouse Tumor Model Establishment and Liposome Injection. Three experiments have been carried out to accomplish Task 1. In the first experiment, 45 female mice at 12 weeks of age were obtained and quarantined for 2 weeks before sc injection of 10^5 cells in 100 μ l in the right flank. The body weight of the animals was recorded every 2-3 days. Tumors became visible

around 8 days after 4T1 cell inoculation. The tumor size was measured with a caliper every 2-3 days, and the tumor volume was estimated using the formula $(L \times W^2)/2$ (10-12). Two weeks after the inoculation of 4T1 cells, tumors were well established in 40 mice. The animals were divided into 3 groups: a group with no injection as control, a group with injection of 10 μ l liposome-encapsulated clodronate, and a group with injection of 10 μ l liposome-encapsulated PBS (control liposome) into the tumors. At intervals of 1, 3, 5, and 8 days after liposome injection, tumors were removed, fixed in buffered formalin for immunohistochemical staining with macrophage-specific marker to quantify the number of infiltrating macrophages.

In the second experiment, 28 female mice were obtained and 4T1 cells were injected as in the first experiment. The body weight and tumor size were measured every 2-3 days. Two weeks after the inoculation of 4T1 cells, tumors were well established in all mice. The animals were divided into 3 groups: a group with no injection as control, a group with injection of 30 μ l liposome-encapsulated clodronate at 2 sites on the tumor (15 μ l at each site), and a group with injection of 30 μ l liposome-encapsulated PBS (control liposome) into the tumors. At intervals of 3, 7, and 9 days after liposome injection, tumors were removed, fixed in buffered formalin for immunohistochemistry to quantify infiltrating macrophages.

In the third experiment, a total of 20 mice were obtained and 4T1 cells were injected as in the previous experiments. The body weight and tumor size were measured every 2-3 days. Tumors became visible around a week and were well established 2 weeks after inoculation. The mice were divided into 2 groups: a group with no injection as control, and a group with intratumoral injection of 60 μ l liposome-encapsulated clodronate at 3 sites on the tumor (20 μ l at each site). At intervals of 1, 3, and 7 days after liposome injection, tumors were removed, fixed for immunohistochemistry to quantify infiltrating macrophages.

Immunohistochemistry. Formalin-fixed tumor tissues from 2 experiments (experiments 2 and 3) were embedded in paraffin and sectioned into 4-8 μ m sections. The tumor sections were deparaffinized and the antigen was retrieved by pressure cooking. The sections were then incubated with an antibody to mouse macrophage-surface marker F4/80 or Mac-1, and stained using the Vectastain ABC Kit according to manufacturer's recommendation. The tumor sections were counter stained with hematoxylin.

Chalkley Counting. Macrophages infiltrated into the tumor were quantified using a 25-point array Chalkley Counting Reticle (Klarman Rulings, Inc.) and an Olympus microscope at 250X magnification. 3 "hot spots" were selected in each tumor section for quantification. 2 sections were counted for each tumor.

2. Results

Three experiments have been carried out to examine the effect of clodronate on tumor growth. In all 3 experiments, tumors became apparent approximately 1 week after 4T1 cell inoculation, and well established in 2 weeks with an average volume between 0.2-0.6 cm^3 . Figure 1 shows that injections of liposome-encapsulated clodronate (A, 10 μ l/tumor; B, 30 μ l/tumor; C, 60 μ l/tumor) had no significant effect on the growth of the tumor. In both control and clodronate injected mice, the tumors continued to grow to 3-5 times of the size at the time of liposome injection.

As there was no effect of liposome-encapsulated clodronate on tumor growth, tumor tissues from two experiments in which high dosages of liposome were used (experiments 2 & 3) are processed for immunohistochemistry and Chalkley Counting. Consecutive tumor sections were

used for comparison in immunohistochemical staining. Tumor sections were stained with 2 antibodies specific to macrophage cell-surface marks, F4/80 and Mac-1. Figure 2, Part I A & B were from consecutive tumor sections to determine specificity of the primary antibody used (anti-F4/80). Part II A & B were from consecutive sections to determine specificity of anti-Mac-1 used. Part I C-F were consecutive tumor sections of Part II C-F, respectively. Results showed that both antibodies had similar staining pattern in consecutive tissue sections. Most infiltrating macrophages were observed around the periphery of tumors with a few clusters of macrophages in the center region. However, no apparent difference in the level of infiltrating macrophages was observed between control and clodronate-injected tumors.

The infiltrating macrophages were quantified by Chalkley counting. Results showed no significant difference in the number of macrophages between control and clodronate-injected tumors (Figure 3).

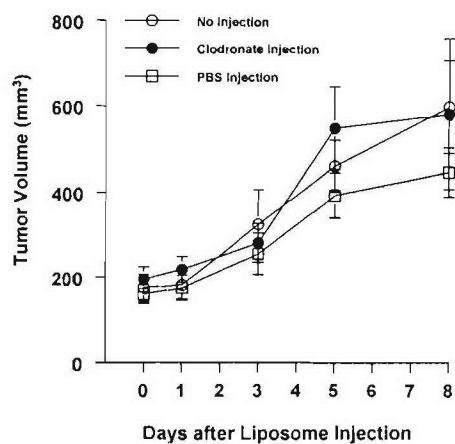


Figure 1A. (Experiment I) Tumor growth after injection of Clodronate-encapsulated liposome. 40 tumor-bearing mice were divided into 3 groups and treated with no injection, clodronate injection (10 μ l/tumor), or PBS injection, respectively. Day 0 marks the day of injection. Tumor volume was determined as described in the text. Numbers of animals in each group at each data collection day are shown in Table 1A. Results are presented with mean \pm S.D.

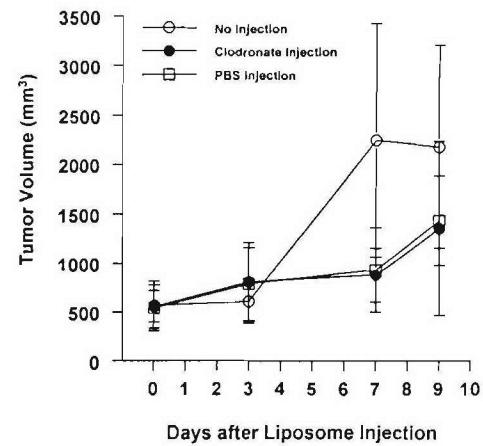


Figure 1B. (Experiment II) Tumor growth after injection of Clodronate-encapsulated liposome. 28 tumor-bearing mice were divided into 3 groups and treated with no injection, clodronate injection (30 μ l/tumor), PBS injection, respectively. Day 0 marks the day of injection. Tumor volume was determined as described in the text. Numbers of animals in each group at each data collection day are shown in Table 1B. Results are presented with mean \pm S.D.

Table 1A. Numbers (n) of animals in each group

	n				
	Day 0	Day 1	Day 3	Day 5	Day 8
No Injection	14	14	10	7	4
Clodronate Injection	13	13	10	7	4
PBS Injection	13	13	8	5	2

Table 1B. Numbers (n) of animals in each group

	n			
	Day 0	Day 3	Day 7	Day 9
No Injection	9	9	6	3
Clodronate Injection	9	9	6	3
PBS Injection	10	10	7	4

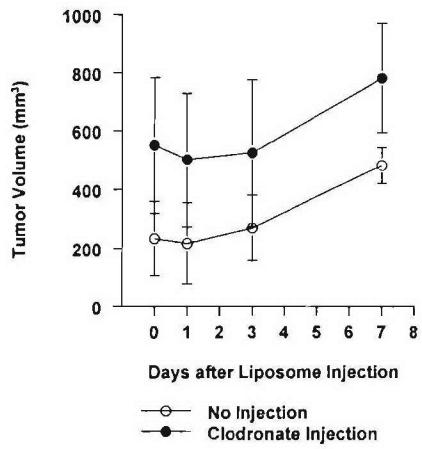


Figure 1C. (Experiment III) Tumor growth after injection of Clodronate-encapsulated liposome. 20 tumor-bearing mice were divided into 2 groups and treated with no injection or clodronate injection (60 μ l/tumor), respectively. Day 0 marks the day of injection. Tumor volume was determined as described in the text. Numbers of animals in each group at each data collection day are shown in **Table 1C**. Results are presented with mean \pm S.D.

Table 1C. Numbers (n) of animals in each group

	n			
	Day 0	Day 1	Day 3	Day 7
No Injection	10	10	7	4
Clodronate Injection	10	10	7	4

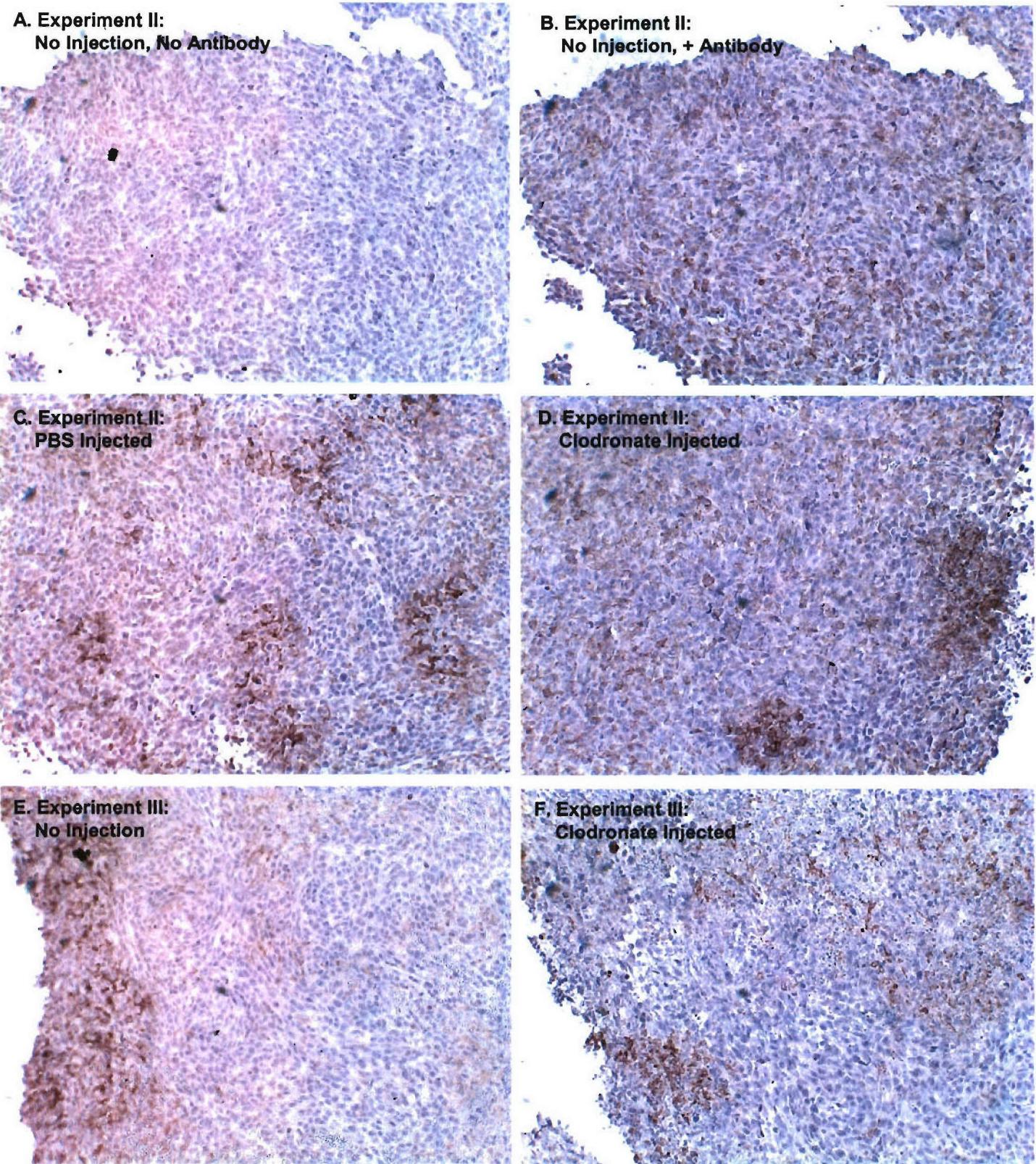
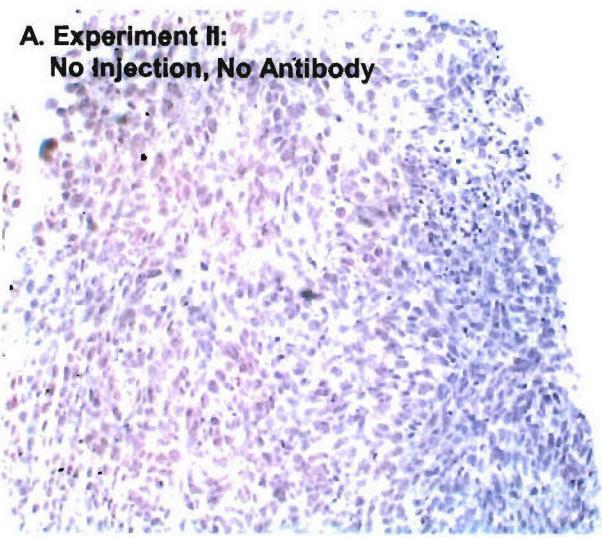
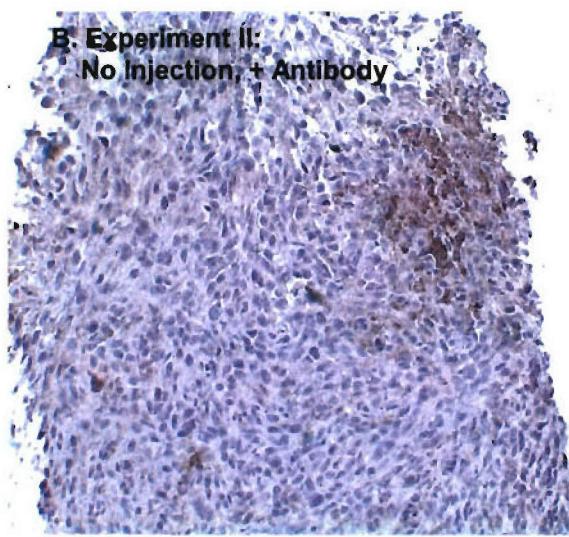


Figure 2, Part I: Tumor Sections Stained with Anti-F4/80 Antibody

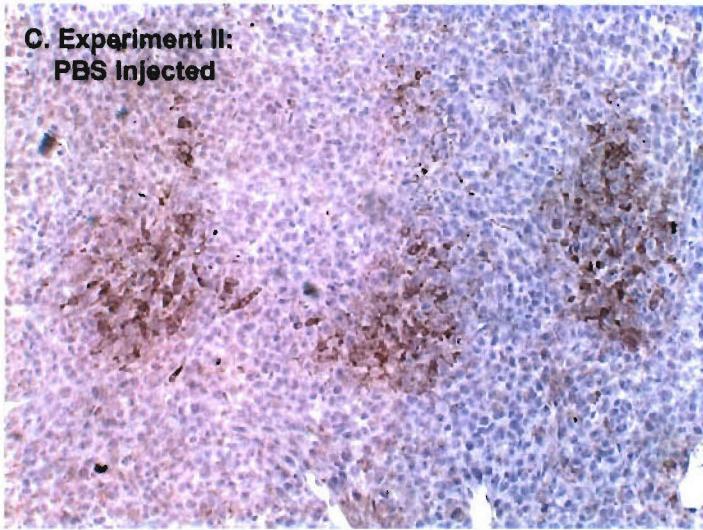
A. Experiment II:
No Injection, No Antibody



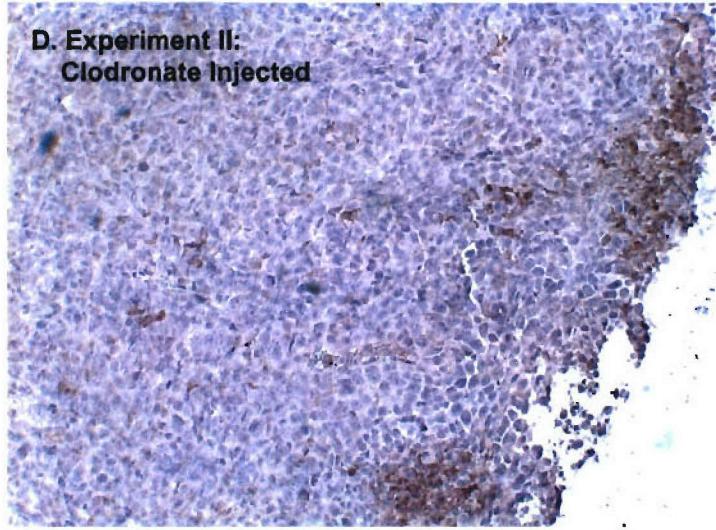
B. Experiment II:
No Injection, + Antibody



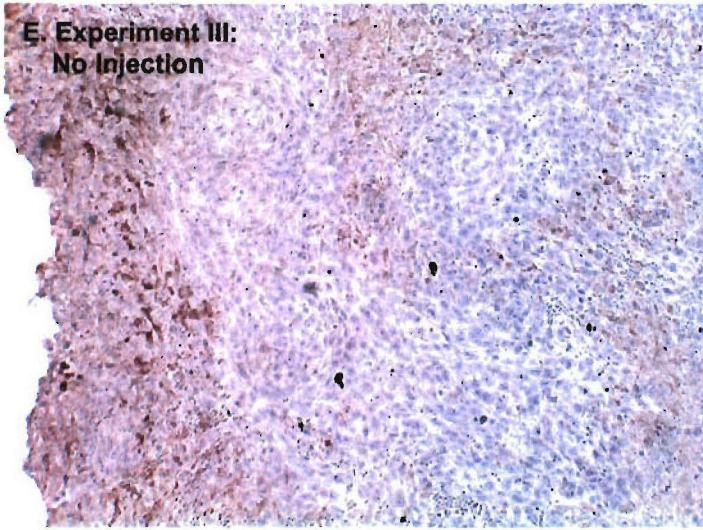
C. Experiment II:
PBS Injected



D. Experiment II:
Clodronate Injected



E. Experiment III:
No Injection



F. Experiment III:
Clodronate Injected

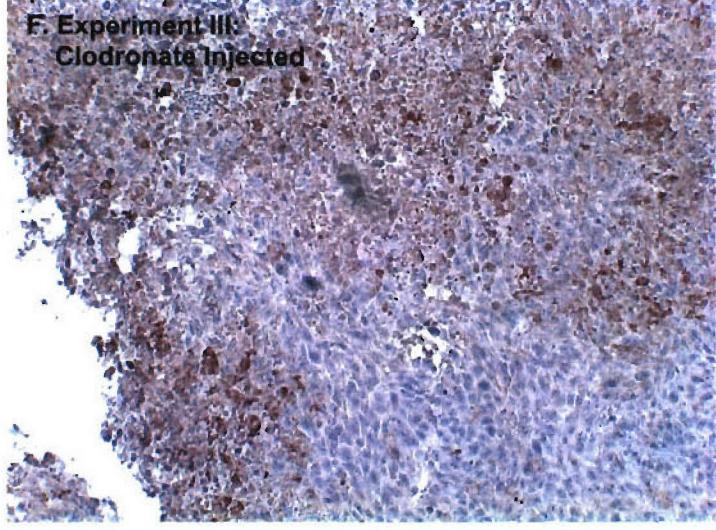
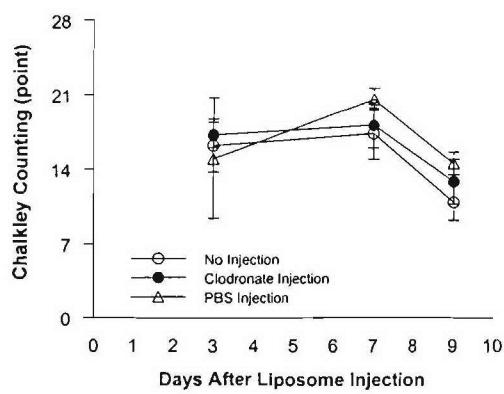


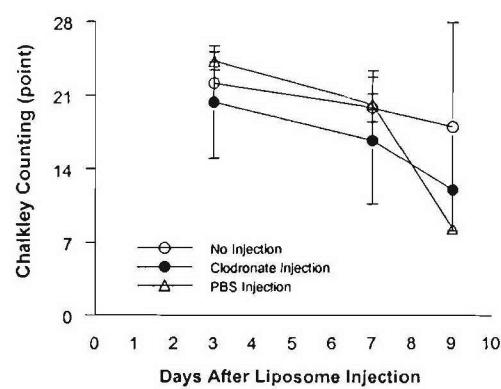
Figure 2, Part II: Tumor Sections Stained with Anti-Mac-1 Antibody

Figure 2. Immunohistochemical staining of macrophages in tumor tissue. The tumor tissues collected from the 2 experiments (Experiments 2 and 3 described in Figure 1) were embedded in Paraffin and sectioned into 4-8 μ m slices. The tumor sections were fixed, deparaffinized, and pressure-cooked to retrieve antigen. The sections were then stained with an antibody to mouse macrophage-surface marker F4/80 or Mac-1. The tumor sections were counter-stained with hematoxylin. Consecutive tumor sections were used for comparison. Part I A & B were from consecutive tumor sections. Part II A & B were from consecutive sections. Part I C-F were consecutive tumor sections of Part II C-F, respectively. **Part I:** tumor sections stained with an antibody to mouse macrophage marker F4/80. **Part II:** Tumor sections stained with an antibody to mouse Mac-1 (CD11b).

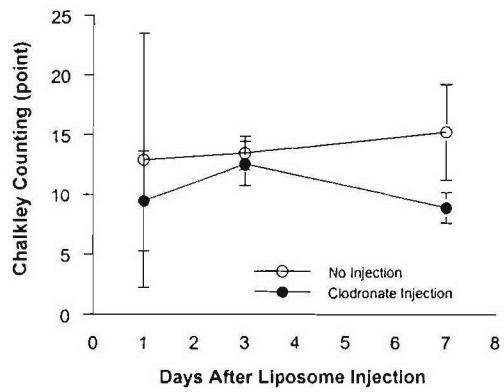
A. Experiment II: Stained with Anti-F4/80



B. Experiment II: Stained with Anti-Mac-1



C. Experiment III: Stained with Anti-F4/80



D. Experiment III: Stained with Anti-Mac-1

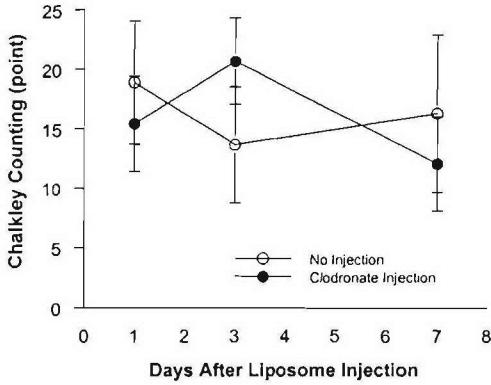


Figure 3. Quantification of infiltrating macrophages. Macrophages infiltrated into the tumors were quantified using a 25-point array Chalkley Counting Reticle and Olympus microscope at 250X magnification. 3 "hot spots" were selected in each tumor section for quantification. 2 sections were counted for each tumor, one stained with anti-F4/80 and the other anti-Mac-1 (see Figure 2). Tumors from 2-4 mice in each group were examined. Values depict averages of each group \pm S.D.

3. Discussion

Liposome-encapsulated clodronate is a potent macrophage-depleting agent. A single injection of 100 μ l intravenously into mice is sufficient to deplete most of the hepatic and splenic macrophages (6). However, in this study we were unable to deplete TAM in a murine breast cancer model even after intra-tumoral injection of as much as 60 μ l of liposome-encapsulated clodronate. The reason for this failure is not clear. It is possible that the injected liposomes did not diffuse to areas where macrophages reside in the tumor. We observed that most TAM were located at the periphery of the tumor. In the Experiment III, we injected liposomes at three different locations in the tumor to increase the coverage of the tumor. However, we observed no effect in depleting TAM. Whether further increases in the dosage of liposome-encapsulated clodronate will have any effect in TAM depletion requires further investigation.

KEY RESEARCH ACCOMPLISHMENTS

- Established breast cancer model in BALBc mice.
- Treated tumor-bearing mice with liposome-encapsulated clodronate.
- Established optimum conditions for immunohistochemical staining of tumor tissue for macrophage quantification.
- Established Chalkley Counting protocol for macrophage quantification.

REPORTABLE OUTCOMES

None

CONCLUSIONS

The purpose of this project is to determine whether *in vivo* depletion of tumor-associate macrophages (TAM) is feasible and if it is feasible, what is the effect of TAM-depletion on the growth of breast cancer and its response to cancer chemotherapy. Using a murine subcutaneous breast cancer model, we have demonstrated that intra-tumoral injection of up to 60 μ l of liposome-encapsulated clodronate had no effect on either the tumor growth or the number of macrophages infiltrated into the tumors. Whether liposome-encapsulated clodronate at a dose higher than 60 μ l will have any effect needs further investigation.

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APPENDICES

None